

Salmonella in Feed and the Relationship to Strains Found in Swine Fecal and
Environmental Samples

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Abstract:

Salmonella is a zoonotic foodborne pathogen with several common serotypes which can be ingested through contaminated meat. Feed, environmental, and fecal samples were all found to contain *Salmonella* in a previous study conducted by the IDMEL. The purpose of this new study was to identify and characterize the possible role of *Salmonella* in the feed, and its phenotypic and genotypic relationship with fecal and environmental samples. A total of 280 isolates were tested and categorized based on serogrouping, genotyping, antimicrobial susceptibility testing, and PFGE DNA fingerprinting. The results demonstrated five genotypic clusters of highly similar isolates. Four of these clusters showed a genotypic relationship between the isolates of feed origin and those of fecal origin. The four clusters identified with feed and fecal isolate relationships were serogroup B with AmStTeKm resistance, serogroup C with SuTe resistance, serogroup B with Te resistance, and serogroup E with Te resistance. The study also identified a high proportion of multi-drug resistant isolates. The most common multi-drug resistant patterns were SuTe (20.4%), Te (28.2%), and AmStTeKm (12.9%). Serogroups C (26.8%), E (13.6%), and B (42.5%) were found to be the most common. These patterns were found in the feed and fecal samples from the same barn at the same collection time. The significance of finding multidrug resistant, epidemiological connections between the isolates indicates feed as an important source of contamination. Therefore targeted intervention measures may be designed to reduce transmission and contamination of *Salmonella*, particularly multi-drug resistant strains, into the food supply.

Introduction:

Salmonella organisms are important foodborne pathogens of public health concern and pork plays an important role in the transmission of *Salmonella* to humans through the food chain. It is well known that pork is an important food source and contributes to the dietary intake of protein and other nutrients. In some societies it holds cultural significance. *Salmonella* can be ingested through contaminated food and food products including pork. Reports indicate that 6 - 9% of foodborne *Salmonella* comes from pork consumption (Torrance, Isaacson, 2003). The Center for Disease Control found that in 1973-1987 *Salmonella* was responsible for 42% of the outbreaks and 51% of cases, while 11% of *Salmonella* outbreaks were related to pork ingestion (Harris, Fedorka-Cray., 1997). In 2002, it was estimated that *Salmonella enteric* caused 1,412,498 cases of human illness, 16,430 hospitalized and 582 deaths per year in the United States (Crump, Griffin, Angulo, 2002). Studies have attempted to trace the source of the *Salmonella* in pigs to three distinct sources; the feed, the barn environment and swine interaction through their fecal waste. However, the data is still very limited to reach to a conclusion.

Animal feed is possibly the earliest way to contaminate animals in food production systems. Studies have shown that animal feed is often contaminated with some form of non-Typhi serotypes of *Salmonella enteric* and these pathogens may later colonize the animals (Crump, Griffin, Angulo, 2002). A significant concern when monitoring animal feed for possible pathogens is the amount of feed produced, number of feed companies and mills, and the possibility of contaminated feed crossing state and international borders. In the United States, in 2000, 119 million tons of feed was produced and the leading 85 companies in the business were utilizing a little fewer than 850 mills and producing a total of slightly less than 44 million tons of

feed (Crump, Griffin, Angulo, 2002). A 1993 FDA study found in 78 plants that produced animal protein-based feeds 56% of the 101 samples were contaminated with *S. enteric* (Crump, Griffin, Angulo, 2002). In the same study, 46 feed mills that produced vegetable protein-based feeds, only 36% of the fifty samples were contaminated (Crump, Griffin, Angulo, 2002). A study from Curtain in 1984 found that 77% of *Salmonella* colonization in animals was similar to their feeds (Jones, Richardson, 2002). Shirota et al. group found in 2001, in a poultry study, that the *S. enteritidis* strains in their commercial eggs were identical to the strains in the feed according to Pulse Field Gel Electrophoresis (PFGE) analysis (Jones, Richardson, 2002).

There are currently various laboratory techniques that have been proven to be accurate and helpful in identifying epidemiological connections. One technique is PFGE, as utilized above in the Shirota et al study. PFGE is a genotypic method which allows researchers to compare DNA between various isolate strains and determine similarities. Currently, PFGE, is the most common DNA fingerprinting technique for foodborne pathogens (Gebreyes, Altier, Thakur, 2006). It has been used as one of the best diagnostic tools in genotyping foodborne diseases. Another tool for identifying similarity between *Salmonella* isolates is phenotyping. Looking at specific isolates phenotypes and genotypes, researchers can identify common strains and create clusters. Observing drug resistance patterns is another technique for identifying the relationship between various samples. In the current study, we compared and clustered isolates by phenotypes, genotypes and drug resistance patterns.

Fecal, feed and swab samples were collected from pigs in North Carolina for another project (USDA-NRI 2007-01778) to study the role of specific classes of biocides and heavy metal micronutrients on the occurrence and persistence of multidrug resistant *Salmonella* in swine production environment. Some of the feed samples collected for that project contained

Salmonella. However, little was known about the identity and association of the *Salmonella* found in the feed with those detected in swine feces and from barns. A question of interest was to investigate whether the feed served as a source of contamination to the swine operations in those farms included in the biocide project.

Objective:

The project objective was to evaluate the feed, fecal and swab samples in detail, and to identify any association of the *Salmonella* found in the feed with those in feces and environmental samples, from the same barns, using various phenotypic and DNA finger printing approaches.

Methods and Techniques:

Samples collected from several pig barns (n=36) in North Carolina were processed for *Salmonella* isolation and identification. Feed samples (about 100 g) were taken directly from all the feeders in the finishing barns and aggregated into one pooled sample per barn. Ten drag swab samples per barn were collected before and after disinfection of barns, before pigs were placed in each selected barn. A total of 48 fecal samples were collected from pigs in each barn (one sample per pen) per rectum using gloved hands. Approximately 25 grams were collected for multiple different analyses and a 200 µg specimen was frozen at –20°C for DNA extraction. *Salmonella* was isolated and identified following conventional culture methods as described in detail below. *Salmonella* suspect colonies were further identified and classified by biochemical tests, antimicrobial susceptibility testing and serogrouping, and pulsed-field gel electrophoresis (PFGE).

Salmonella isolation and identification:

Once feed/fecal samples arrived from North Carolina, 10 grams were weighed and 90 ml of Buffered Peptone Water (BPW) was added to the cup and mixed well. To each swab sample, 90 ml of BPW was added and mixed well. All samples types were incubated at 37°C for 16-20 hours. On the second day, 100 microlitres of suspension from the specimen samples were transferred to tubes containing 9.9 ml of Rappaport Vassiliadis (RV) media, a salmonella selective media, and incubated at 42°C for 24 hours. A loopful of the culture from the RV was streaked on to Xylose-Lysine-Tergitol TM 4 (XLT4) agar plate and incubated for 24 hours at 37°C. Presumptive *Salmonella* positive colonies on XLT agar plate colonies were transferred to

TSI by a blunt end loop, one colony was chosen to make a stab to TSI agar slant and then streaked on urea agar slant for growth as well. This was repeated for three colonies per each positive plate and incubated for 24 hours at 37°C. *Salmonella* positive colonies were then streaked from the TSI onto the Luria-Bertani agar (LB) and incubated for 24 hours at 37°C. All tubes were labeled based on database number, isolates prepared for freezing and LB tubes were parafilmed and stored at room temperature.

Once samples had been tested and identified positive for *Salmonella*, as described above, they were aseptically transferred from TSI to LB slants and incubated at 37°C overnight and prepared for antimicrobial susceptibility testing, serogrouping and genotyping. The samples were transferred from LB to cryovials and stored at -80°C. After cryovials were prepared, a loopful of sample from the same LB slants was plated on LB/Mueller Hinton agar.

Serogrouping:

Serogrouping of *Salmonella* isolates was performed through slide agglutination using commercially available polyvalent O and group specific antisera (Mira Vista, Copenhagen, Denmark). One drop of antisera was placed onto a cleaned glass slide and a part of the colony to be tested was placed in the drop for up to a minute to obtain a homogenous suspension. The slide was rocked gently for 1 to 2 minutes and observed again against a dark background with a magnifying glass to see if the reaction was positive. This was repeated with polyvalent and monovalent sera and included positive controls.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility of *Salmonella* isolates from feed, drag swabs and fecal samples was tested to a panel of 12 antimicrobials using the Kirby-Bauer disc diffusion method

as recommended by the Clinical Laboratory Standards Institute, CLSI (NCCLS, 2002). The following antimicrobials with the respective disc concentrations were used: ampicillin (Am; 10µg), amoxicillin-clavulanic acid (Ax; 30µg), amikacin (An; 30µg), ceftriaxone (Ce; 30µg), cephalothin (Ch; 30µg), chloramphenicol (Cl; 30µg), ciprofloxacin (CIP; 5µg), gentamicin (Gm; 10µg), kanamycin (Km; 30µg), streptomycin (St; 10µg), sulfisoxazole (Su; 250µg) and tetracycline (Te; 30µg). Briefly, 4 to 5 isolated colonies were taken from the LB/MH plates, with a sterile cotton swab touching the top of each colony and transferred to tubes containing 4 to 5 ml of sterile saline solution (0.9% i.e. 8.5g NaCl in 1000 ml dist. water). The inoculum was emulsified and then adjusted the turbidity to McFarland 0.5. After 15 min, a sterile cotton swab was dipped into the suspension and rotated several times until excess inoculum was removed from the swab. The inoculate swab was struck over sterile agar surface and this was repeated two more times to evenly distribute inoculums. The disks were dispensed to agar surface with the disk dispenser and with sterile forceps each disk was gently pressed down to ensure contact with agar surface and then incubated for 16 to 18 hours at 37°C. The plates were examined thoroughly and the zones of inhibition measured to the nearest millimeter with a sliding calipers ruler from the back of an inverted petri plate. Measurements began after controls were checked and plates were read by a minimum of two people checking the results. Results were classified as either susceptible, intermediate, or resistant, and all differences were recultured and reread by a third person. *Escherichia coli* ATCC 25922, *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Each resistant isolate, which showed resistance to three or more classes of antimicrobials, was classified as multi-drug resistant (MDR).

Genotyping (DNA fingerprinting):

The Pulsed-field gel electrophoresis (PFGE) was used for genotyping the isolates originating from swine feed, feces and barn swabs. PFGE was performed according to the Centres for Disease Control and Prevention (CDC) PulseNet protocol [Ribot et al., 2006]. Briefly, *Salmonella* isolates were grown overnight on Luria-Bertani (LB) agar (Becton Dickinson, MD). Bacterial cell concentration was adjusted by diluting with sterile cell suspension buffer to the OD value 1.3 - 1.5 at 610 nm wave length. Agarose-embedded cells were lysed and intact genomic DNA was digested with 50U of *XbaI* restriction enzyme (New England Biolabs, Ipswich, MA) for at least 2 hours at 37°C. The fragments were then separated by CHEF-DR[®] III Pulsed-Field Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) with the following conditions and reagents: 1% SeaKem Gold agarose (FMC BioProducts, Rockland, Maine) in 0.5% Tris-borate EDTA buffer, temperature: 14°C; voltage: 6 v/cm; run time: 18 hours with switch times ranging from 2.2 to 63.8s. *Salmonella enterica* serovar Braenderup H9812 was used as a molecular reference marker. The gels were stained with ethidium bromide and the DNA bands were visualized under UV trans-illumination (Gel Doc[™] 2000, Bio-Rad Laboratories, Hercules, CA) and PFGE gels were analyzed by Bionumerics software V. 4.61 (Applied Maths NV, Belgium)..

The major materials needed for this project were different types of media including BPW, RV, TSI, TSA, LB, and MH, antimicrobial disks, antisera for serogrouping, various reagents for PFGE. Additional materials required included the numerous incubators, freezers, PFGE machine, loops, pipettes and other basic lab tools necessary for sterile technique.

Analysis:

The serogrouping, antimicrobial susceptibility and PFGE genotyping results were qualitatively compared for similarities between the *Salmonella* found in the feed, environmental and fecal samples. For genotypic analysis, the Bionumerics software (version 4.6) was used. The resistance between all of the different samples was assessed. A database was created.

Results:

TABLE 1. Phenotypic characteristics of *Salmonella* isolated from swine feed, barn floor swabs, and feces from the same barns, farms, and production systems^d

Farm ^b	Stage ^c	Replicate ^a	Phenotypic characteristics of <i>Salmonella</i> isolates (no. of samples) ^e					
			Feed (30)		Swabs (71)		Feces (179)	
			Serogroup(s)	R type	Serogroup(s)	R type	Serogroup(s)	R type
RW7	F1	R1	C	Su Te (3)	E	Te (3)	C C A to I B E E D C C B B E B C A to I A to I	St Su Te (1) Su Te (36) Am Te (6) Te (2) Te (15) Su Te (3), St Su Te (1) St Su Te (2), Te (1) Te (2), St Su (2), Su (6) Am St Su Te Km (1) Am Cl St Su Te (4), Su (1) Am Su Te Km (3) Te (5) Am Cl St Su Te/Ax (5) Su Te (3) Te (1), Am Te (2) Am Su Te (1)
RW6	F2	R1	E	Te (3)	A to I	Te (1)		
RW4	F1	R1	A to E	Am St Te (3)	B	Am Cl St Su Te (4)		
TT2A		R1	A to I	Pansusceptible (3)	B B C B B	St Su Te (14) Am Cl St Su Te (3) St Su Te (1) St Su Te (4) Am St Te Km (4) Am St Su Te Km (1)		
BH3		R1	A to I	Te (3)				
RW4	F1	R3	B	Te (3)	E B	Te (1) St Su Te (2)	B C C E E B B A to E A to I A to I E	Te (20) Su Te (1) St Su Te (2) Am Te (2) Am Su Te (1) Te (5) St Su Te (6) St Su Te (1) Su Te (7) Te (1) Am St Su Te (1)
RW6	F1	R3	B	Te (3)	A to I	St Su Te (4)		
GO2A	F2	R3	B	Am Cl St Su Te (3)	C C C G E	St Su Te (12) Su Te (4) St Su (1) Te (3) Te (6)		
FF12	F2	R3	B	Am St Te Km (3)			B	Am St Te Km (23)
TE6A	F2	R4	B	Am St Te Km (3)	E	Te (1)	B (F2) B	St Su Te (3) Pansusceptible (3)

Figure 1. Antimicrobial Abbreviations and Disc Potencies: Ampicillin (Am; 10µg), Amoxicillin-clavulanic acid (Ax; 30µg), Amikacin (An; 30µg), Ceftriaxone (Ce; 30µg), Cephalothin (Ch; 30µg), Chloramphenicol (Cl; 30µg), Ciprofloxacin (CIP; 5µg), gentamicin (Gm; 10µg), kanamycin (Km; 13 30µg), streptomycin (St; 10µg), sulfisoxazole (Su; 250µg), and Tetracycline (Te; 30µg).

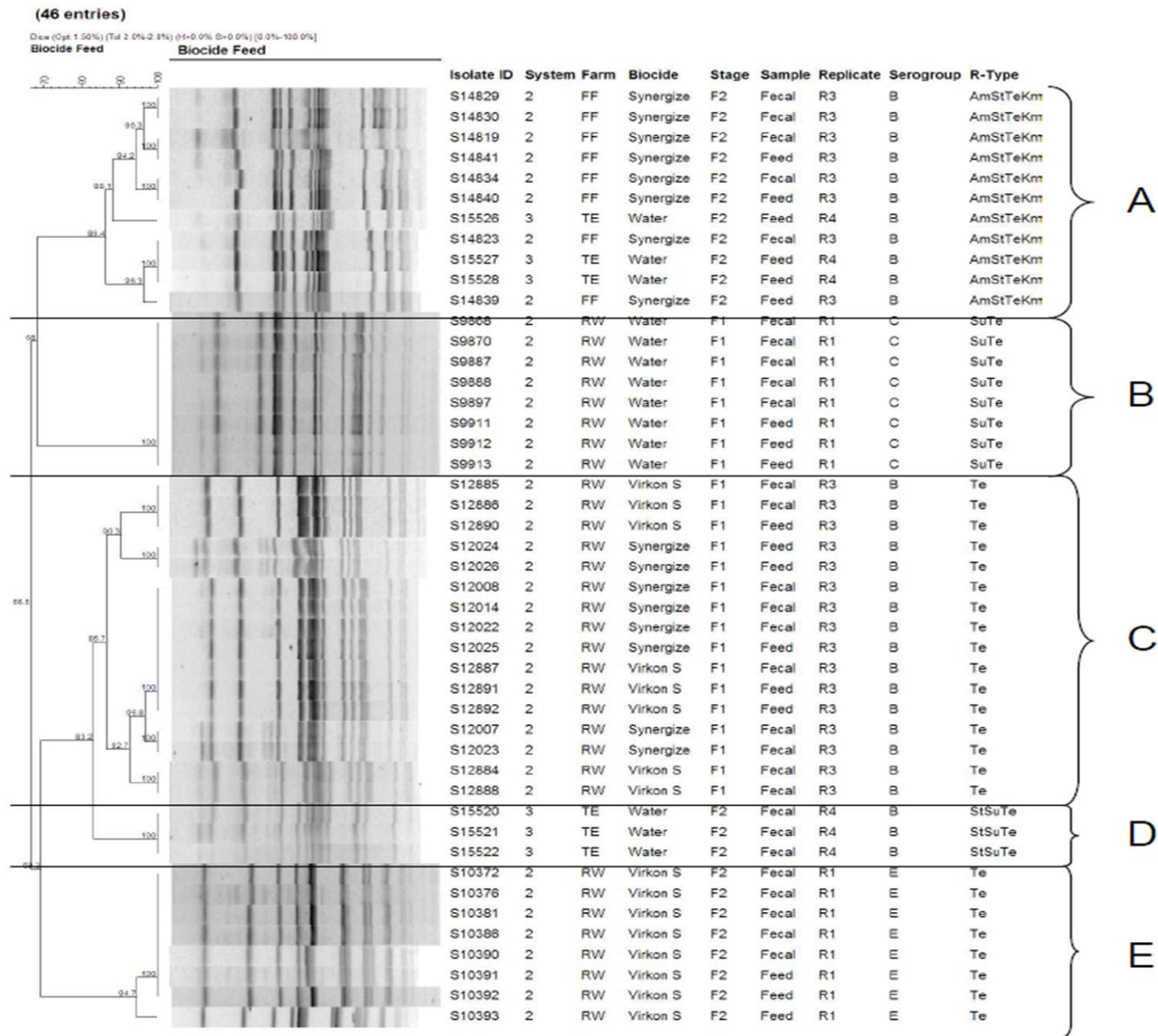


FIG. 2. Dendrogram of PFGE profiles of *Salmonella* recovered from swine feed and fecal samples and their association with sources (farm, production stage, and replicate), serogroups, and antimicrobial resistance patterns. The following are terms used in the dendrogram: system, swine production system (three vertically integrated production systems were included in the study, production systems 1, 2, and 3); farm, symbols FF, TE, and RW are used to represent farm names; stage, stage of production (F1, early finishing stage; F2, late finishing stage [market age]).

Two hundred seventy five isolates were collected from 8 different barns, examined, and pooled. There were 3.6% positive *Salmonella* isolates. *Salmonella* positive feed was identified in 8 of 36 barns, 22%. Environmental samples were also taken pre and post disinfection and found to be 15.3% (206/1,350) and 7.5% (101/1,350) positive for *Salmonella*. Fecal samples were taken during two different stages of production; market age and early finishing stage. The pigs at the market age were 7.4% (392/5,321) positive, significantly less than the early finishing stage which had a 17.2% (1,180/6,880) positive for *Salmonella*.

Figure 1 (above) shows the phenotypic, genotypic and drug resistance patterns of the 275 isolates from the environmental swabs, feces and feed. From the positive feed isolates, 90% (27/30) showed drug resistance to at least one of the 12 tested antibiotics. Forty four percent (12/27) of those isolates were multidrug resistant (MDR, resistant to three or more antibiotics). Multidrug resistance was found in all three types of samples; feed, environmental swabs, and feces. The most common type of MDR was AmStTe/Km, 40%, and the most common type of drug resistance was to tetracycline only, 40%. The proportion of isolates tested that were MDR was significantly higher in both feces and environmental samples compared to feed samples.

To determine a closer genotypic relationship between samples, 46 phenotypically similar isolates were selected for PFGE DNA fingerprint analysis. The samples were selected based on similar source (farm they came from, stage of production, and replication cycle), antimicrobial resistance pattern, and serogroup identification. The breakdown of these isolates was 18 of the 30 feed samples and 28 of the 179 fecal samples. The results are shown above in Figure 2. Environmental samples, swabs both pre and post disinfection, were not included in the PFGE analysis because they did not share any antimicrobial resistance patterns and serogroups with the

feed isolates. Samples were clustered into five groups (A, B, C, D, and E) based on the PFGE DNA fingerprinting analysis results. These clusters exhibit an 85% genetic relatedness threshold.

Discussion:

MDR patterns between feed and fecal samples were very similar. The most common type of resistance for feed was AmStTe/Km (40%), and then Te (40%). However for fecal it was AmStKm (21%) and StSuTe (9%). The most common MDR for the environmental samples was StSuTe (52.1%). Based on serogroup identification, 50%, 45.1% and 42% of the feed, environmental and fecal samples respectively were group B. Serogroup B is the most common serogroup for *Salmonella* (Gebreyes, W. A., C. Altier, and S. Thakur. 2006). Of the feed and fecal samples in the current study, 50% and 55.1% respectively shared both a serogroup and antimicrobial resistance pattern.

The PFGE DNA fingerprint results were consistent with the previous serogrouping and antimicrobial resistance results. Five clusters (A, B, C, D and E) with an 85% similarity were identified between the feed and fecal samples. In four of the five (A, B, C, and E) clusters, there was a connection between the feed and fecal isolates showing a possible continuation of contamination from feed to feces. There were no feed samples identified in cluster D. From the five clusters, only cluster A had samples from two different production systems. The isolates had the same serogroup and antimicrobial resistance but were from different farms. This shows the genotypic relationship between production systems and between feed and fecal samples. The fecal and feed isolates were indistinguishable between the two types of samples in each cluster in the PFGE results. This connection shows that as this study hypothesized, feedstuff was a possible source of contamination in swine production. PFGE is limited in its genotypic uses and the amplified fragment length test (AFLT) may be a better test with higher throughput.

From the phenotypic characteristics, the PFGE, serogroup, and antimicrobial resistance patterns, the findings show there was more than 50% clonality between the feed and fecal samples. The high percent of clonality between the feed and fecal samples indicates that there was an epidemiological relationship between the isolates.

Salmonella can contaminate and be found in any organic feed ingredient. A study from 2004 found *Salmonella* in the dust released while feed was being produced. The dust that was studied always reflected higher contamination than the feed being processed at the time of sample collection (Jones, Richardson, 2002). This is a concern because pellet dust is often overlooked when attempting to identify possible sources of contamination. The dust could easily accumulate in a mill and with any form of ventilation, especially by the pellet coolers that pull around 5,000 cfm of air, dust could reinfect “sanitized” or already heat treated feeds (Jones, Richardson, 2002).

Several studies have looked at better ways of producing food animal feed and most likely sources of contamination to cut down on pathogen spread. A study from 2004 found that pelleting feeds reduced *Salmonella* isolation by 50% in their study (Jones, Richardson, 2002). For pelleting to be effective at decreasing contamination in feeds it must reach a high enough temperature. Veldman et al. in 1995 found that if the feed is pelleted, the temperature must be greater than 80 degrees centigrade to reduce *Salmonella* levels (Jones, Richardson, 2002). *Salmonella* will be able to survive and still be infective if a high enough temperature is not reached. Another Danish study found that a majority of the feed contamination came from the pathogens growing during the manufacturing process because of the moist, dry feed production conditions (Jones, Richardson, 2002). The majority of the moisture was identified to come from the pellet cooler.

Conclusion:

Salmonella is a zoonotic pathogen that can be contracted by ingesting contaminated food including pork and pork products. There are many sources of *Salmonella* infection of swine including contaminated feed, barn environment, rodents, insects, humans, and contaminated feed and feedstuffs. From the results of this study, *Salmonella* in the feed isolates collected appears to be clonal with the fecal samples collected and the apparent source of contamination in the pigs themselves. This study also showed the presence of *Salmonella* in feed, feces and in the environment the pigs were living in. The environmental samples in this study did not match the feed samples indicating different sources of contamination. However, there were environmental samples that matched fecal samples, as well as feed samples identical to fecal samples indicating the possibility of both feed and environment as sources of fecal contamination.

Sweden has a “farm to fork” surveillance program in place for various pathogens including *Salmonella*. This surveillance system monitors the feed, animals, finished and packaged food product to track contamination (Crump, Griffin, Angulo, 2002). In the United States, setting up a similar surveillance system for pathogen contamination is essential. This system needs to include a way to trace any contaminated feeds back to the production companies and from there to the original site of contamination (Crump, Griffin, Angulo, 2002).

In 1991 the FDA did pass a *Salmonella* negative policy for feeds but since then it has not been implemented or enforced (Crump, Griffin, Angulo, 2002). The current study suggests the FDA policy should be reconsidered as a means to decrease *Salmonella* contamination in the swine industry.

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